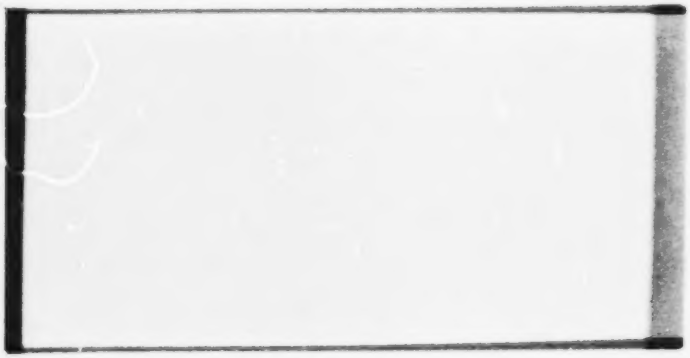
ontario hydro
research division



Named in honour of Dr. W.P. Dobson, the Research Division's director from 1914 to 1953, the building shown on the front cover was first occupied in 1961. Along with the adjacent pump testing complex and the high-voltage, high-current and radioactive materials laboratories, it accommodates much of the research, development and testing work performed in support of the diverse technical needs of Ontario Hydro.

The Research Division's staff is engaged on a wide range of problems relating to the design, construction, operation and maintenance of the power system and to the efficient, economical use of electric power.

Ontario Hydro, Research Division
800 Kipling Avenue,
Toronto, Ontario, Canada
M8Z 5S4

Telex: 06-984-525

Phone: (416)231-4111

91602
new 82-10

COMPLIMENTARY COPY
Please Credit Ontario Hydro Research if
Reproduced in Whole or in Part.

ontario hydro
research division

MICROBIOLOGICAL PROCEDURES - MICROBIAL/ACID RAIN INTERACTIONS

Report No 82-569-K

V.J. Sharpe
Biologist
Biological Research Section
Chemical Research Department

ABSTRACT

It was necessary to develop methods for the isolation and enumeration of physiologically specific groups of bacteria involved in nitrate and sulphate reduction as part of the investigation into microbial/acid rain interactions. Methods were based on monitoring the presence or absence of metabolic intermediates or end products through the use of chromogenic reagents. Optimal parameters for the culture and detection of anaerobic nitrate-reducing and denitrifying bacteria were obtained by combining and modifying microtitre and Most Probable Number techniques. Enumeration of sulphate-reducing bacteria was optimized using a selective culture procedure as part of the Most Probable Number method. Counts of viable bacteria obtained from an acidic podzol using these procedures were: nitrate-reducers 8.13×10^5 cells/g, denitrifiers 4.75×10^4 cells/g, and sulphate-reducers 2.24×10^4 cells/g, which fell within accepted values for these bacteria as determined by different methods.

job	file	date	report no.
740634-529-032	837.08	February 16, 1983	82-569-K

TABLE OF CONTENTS

		Page
1.0	INTRODUCTION	1
	1.1 Reduction of Nitrate	2
	1.2 Reduction of Sulphate	2
2.0	METHODS	
	2.1 Nitrate-Reducing and Denitrifying Bacteria	3
	2.1.1 Medium Constituents	3
	2.1.2 Reagents for Detection of Nitrate and Nitrite	4
	2.1.3 Modification of MPN Technique	4
	2.1.4 Procedures	6
	2.2 Sulphate-Reducing Bacteria	
	2.2.1 Microtitre/MPN Technique	8
	2.2.2 Selective Medium/MPN Method	9
3.0	RESULTS AND DISCUSSION	
	3.1 Nitrate-Reducing and Denitrifying Bacteria	10
	3.2 Sulphate-Reducing Bacteria	14
4.0	CONCLUSIONS AND RECOMMENDATIONS	14
	REFERENCES	16
	APPENDIX A - MEDIA AND REAGENT COMPOSITION	18
	DISTRIBUTION	last page

LIST OF TABLES

		Page
Table 1	Factors for Calculating the Confidence Limits for the Most Probable Number Count	6
Table 2	Details of Substrates and Reagents Used in a Modified Microtitre/MPN Procedure	7

LIST OF FIGURES

Figure 1	Diagrammatic Representation of a Typical Inoculated Microtitre Plate Containing Nutrient Broth Plus 10 mM Nitrate	12

NOMENCLATURE

aerobic	-	of an environment where the partial pressure of oxygen is similar to normal atmospheric conditions.
anaerobic	-	of an environment where there is no oxygen.
assimilatory nitrate reduction	-	typically an aerobic process whereby nitrate is reduced via nitrite to ammonia which is assimilated by the addition of ammonia to a keto acid in a process called amination, to form an amino acid.
denitrification	-	the process of nitrate respiration with gaseous reduced nitrogen forms as the end product.
dissimilatory sulphate reduction	-	the obligate use of sulphate as a terminal electron acceptor for (anaerobic) respiratory metabolism with the elimination of hydrogen sulphide as an end product.
facultative	-	literally means optional, not a fixed or obligate requirement/state.
microaerophilic	-	of an environment where the partial pressure of oxygen is lower than that of normal atmospheric conditions but is not quite an anaerobic environment.
microtitre	-	method used in immunology/virology to determine the concentration of antibodies (or antigens) in a given sample, where the end point is commonly described by the highest dilution which gives a positive serological reaction. Microtitre plates, with 8 by 12 wells, are used in such a determination.
MPN	-	the Most Probable Number technique - a means of estimating viable microbial numbers in liquid culture without counting single cells or colonies manifested on solid growth media.
nitrate-reducing bacteria	-	bacteria which mediate nitrate respiration with nitrite as the end product.
nitrate respiration	-	(dissimilatory nitrate reduction) the use by some bacteria of nitrate as a terminal electron acceptor for (anaerobic) respiratory metabolism under anaerobic or microaerophilic conditions.

obligate	-	refers to any state or condition which is an essential attribute of a given organism.
redox potential	-	an oxidation-reduction potential (E_h). A measure of the tendency of a given oxidation reduction system (redox system) to donate electrons (ie to behave as a reducing agent), or to accept electrons (ie to behave as an oxidizing agent). The E_h of a system is determined by measuring the electrical potential difference in millivolts where negative mV values are indicative of reducing systems and positive values of oxidizing systems.
sulphate-reducing bacteria	-	anaerobic bacteria (genera - *Desulfovibrio* and *Desulfotomaculum*) involved in dissimilatory sulphate reduction.

91601
rev 82-10

ontario hydro
research division

To Mr. F.J. Kee
Director of Research

MICROBIOLOGICAL PROCEDURES - MICROBIAL/ACID RAIN INTERACTIONS

1.0 INTRODUCTION

The work contained in this report is a contributory part of an investigation into the microbiological and chemical equilibria in acid stressed soils. The fate of the major acid rain components, sulphur and nitrogen oxides, is an important element in the determination of the impact of acidic deposition upon the environment. Terrestrial systems were selected for study because a significant proportion of rainfall passes through watersheds before collection as surface waters. During this passage the acid rain components may be 'neutralized' by transformations mediated by microbial activity. Therefore, a knowledge of the level of microbial activity within a model terrestrial system receiving synthetic acid rain, in combination with chemical data, will aid in the interpretation of the fate of sulphur and nitrogen oxides.

The aim of this work was to devise highly selective methods for the isolation and enumeration of physiologically specific groups of bacteria involved in the transformation of sulphur and nitrogen oxides. The present methods for taxonomically typing these bacteria entails the use of extensive biochemical tests which are time consuming and costly. To reduce the amount of manipulation required to obtain numbers of viable bacteria, methods which are highly specific for a particular physiological type had to be devised.

It was decided to develop methods for particular physiological groups of bacteria allied to specific metabolic pathways by determining the presence or absence of metabolic intermediates or end products. To do this a combination of chemical methods and microbiological methods was employed. In order to appreciate the importance and specificity of detecting these metabolic products a brief explanation of the relevant sections of the sulphur and nitrogen cycles is given.

1.1 Reduction of Nitrate

The transformations of the nitrogen cycle are largely a result of microbial activity which supplies nitrogen in a form suitable for plant and animal utilization. The metabolic pathways involved in the conversion of nitrate, the predominant form of oxidized nitrogen in acid rain, are assimilatory and dissimilatory nitrate reduction.

Assimilatory nitrate reduction is typically an aerobic process and can be mediated by a wide range of bacteria, fungi and most algae. The pathway involves the reduction of nitrate, via nitrite and unknown intermediates, to ammonia which the micro-organisms then assimilate by conversion to amino acids. The process may be inhibited by the presence of reduced organic nitrogenous compounds and ammonia.

In contrast, dissimilatory nitrate reduction occurs under anaerobic conditions or in the presence of limited oxygen levels (microaerophilic). There are two distinct pathways in this process, one is anaerobic respiration which is commonly referred to as just nitrate reduction. This term will be applied throughout this report. In this case, bacteria, which normally use oxygen as a terminal electron acceptor, use nitrate as an alternative when conditions are anaerobic and can only reduce nitrate to nitrite. This type of anaerobic respiration is limited because of the build up of toxic nitrite. The second pathway is called denitrification where nitrate is reduced past the nitrite level such that the end products are gaseous. Dependent upon environmental conditions, these gaseous end products may be nitrous oxide (N_2O), nitric oxide (NO), or dinitrogen (N_2), or some combination of the three.

So, the nitrate component of acid rain can be converted to gaseous forms and lost from the soil, or converted to nitrite which in turn causes inhibition of microbial activity due to its toxic nature. The other possibility is the transformation of nitrate to ammonia, which is temporarily immobilized in microbial cells until its release upon cell death and lysis thereby enhancing soil fertility.

1.2 Reduction of Sulphate

The sulphur in acid rain is mainly in its most oxidized state and therefore is amenable to reduction processes for its transformation. A large number of widely differing microbes, fungi, yeasts and bacteria, use sulphate as their sole source of biological elemental sulphur. The process whereby sulphate is fully reduced to sulphide and assimilated by organisms during the synthesis of sulphur

containing amino acids (cysteine and serine) is called assimilatory sulphate reduction. This biochemical pathway is fairly well understood but the aerobic microbes associated with it are difficult to isolate as a group due to their inherent diversity.

The second pathway is that of dissimilatory sulphate reduction whereby the sulphate ion acts as an oxidizing agent for the dissimilation of organic matter. A small amount of the reduced sulphur is assimilated by the organism, but virtually all is released into the external environment as the sulphide ion, usually in the gaseous form of hydrogen sulphide. This process of "sulphate respiration" is only carried out by specialized bacteria which are referred to as "sulphate-reducing bacteria". The two well established genera of the sulphate-reducing bacteria, *Desulfovibrio* and *Desulfotomaculum*, are still not well taxonomically characterized and are difficult to isolate unless the environment has a low redox potential. Sulphate reducers are obligate anaerobes which grow more slowly than many soil organisms, but have a marked capacity for survival. An interesting by-product of their metabolism is bicarbonate, which can be described by the following equation:

$$2 \text{ lactate} + SO_4^{2-} \rightarrow 2 \text{ acetate} + 2\ HCO_3 + H_2S$$

This means that actively metabolizing sulphate-reducers tend to make their surrounding environment more alkaline, unless acid formation by other bacteria is occurring simultaneously. This could have interesting implications in a study of the impact of acid rain on terrestrial environments.

2.0 METHODS

2.1 Nitrate-Reducing and Denitrifying Bacteria

A rapid and accurate method was required for the enumeration of nitrate-reducing and denitrifying bacteria. To reduce manipulation a simultaneous determination of these two bacterial groups was desirable.

2.1.1 Medium Constituents

Past enumeration methods often entailed the inoculation of growth media containing NO_3^- as the terminal electron acceptor and incubation in sealed tubes to exclude air. Researchers have discussed the most appropriate medium for the enumeration of denitrifiers or nitrate-reducers (Valera and Alexander, 1961), but no general concensus seems to have emerged. The medium to be used in this study must be balanced between containing sufficient nitrate to support good growth and not having so much nitrate that it cannot be used up in general nitrate reducing processes. Reports of nutritionally acceptable

nitrate levels vary from 0.2% w/v NO_3^- (Seeley and VanDemark, 1981) to 0.1% w/v NO_3^- (Sirockin and Cullimore, 1969). It is possible that these nitrate concentrations are too high for the requirements of this investigation. In specific studies of populations of denitrifying bacteria in soil, the reported values of nitrate in the media ranged from 0.1% w/v NO_3^- (Alexander, 1965b) to 0.022% w/v (3.5 mM) -0.045% w/v (7.2 mM) NO_3^- (Volz, 1977). Initially, it was decided to test the suitability of 10 mM NO_3^- (that is 0.062% w/v NO_3^-) for this study (see Appendix A).

2.1.2 Reagents for the Detection of Nitrate and Nitrite

There are several possible methods for observing the activity of these bacteria. Essentially the aim is to detect the reduction of nitrate by its disappearance from the growth medium and the consequent formation of nitrite. The criterion for determining whether the micro-organisms are nitrate-reducers or denitrifiers is the persistence of nitrite in the medium. If nitrate has been metabolized and nitrite has been formed then the mediating organisms are classified as nitrate-reducers. If nitrate and nitrite are not present in the medium, then it is assumed that reduction has occurred past the nitrite stage to produce gaseous end products which can only be performed by denitrifiers.

The presence of nitrite in the spent medium can be qualitatively assessed by adding diazotizing-coupling reagents, like the Griess-Ilosvay reagent (Bremner, 1965) or the acidified 1-napthylamine hydrochloride reagent (American Public Health Association, 1975). Both reagents produce a red colour reaction which is easy to detect.

To ascertain whether nitrate remained unreduced in the growth medium, it can be detected indirectly by adding a chemical reducing agent such as zinc powder or cadmium granules. The converted nitrate, now in the nitrite form, can be detected using the previously described reagents. Alternatively, after the addition of the diazotizing-coupling reagent the residual nitrate can be determined directly using Morgan's reagent of acidified diphenylamine, which produces a blue colouration in the presence of nitrate (Morgan, 1930). The relative sensitivities of these approaches and the possible interference when using two colour reactions in the direct determination of nitrate and nitrite were investigated.

2.1.3 Modification of MPN Technique

Culture of bacteria in liquid growth media is a prerequisite for the observation of growth and subsequent addition of chromogenic reagents for the detection of metabolic products. In order to quantify these results the Most Probable Number (MPN) technique was employed. This method permits the estimation of population density without an actual count of single cells or colonies and is a well recognized

method (Alexander, 1965a). The MPN technique is based on a determination of the presence or absence of micro-organisms in several individual aliquots, each of several dilutions of soil, or other material. It is essential that the micro-organisms whose population is to be determined must be able to bring about some characteristic and readily recognizable transformation in the medium into which it is inoculated. It is also necessary that a single cell can produce sufficient growth to cause a transformation. However, in the assessment of positive or negative it cannot be determined whether one or several cells was present originally. On the basis of probability theory, it is possible to calculate, from the numbers of positive and negative tubes receiving a certain quantity of inoculum, the Most Probable Number of micro-organisms in that quantity of inoculum. Multiplying the result by the appropriate dilution factor gives the MPN for the sample. Theoretical aspects of this method are covered by Cochran (1950). For the conversion of numbers of positive/negative tubes to actual bacterial numbers, tables are readily available for a tenfold dilution series with five replicates per dilution (Alexander, 1965a).

There are two major setbacks with the MPN procedure as it stands. Firstly, the determination of the endpoint is not particularly accurate using tenfold dilutions. Secondly, the degree of replication and lengthy dilution series due to unknown bacterial titres results in the use of very large numbers of test tubes and large volumes of medium. Cochran (1950) produced a table of factors to be used in the calculation of 95% confidence limits for MPN values. A compilation of factors, keyed to the rate of dilution and to the number of tubes per dilution, is given below. Multiplication and division of the MPN number by a factor gives the upper and lower limits respectively. It can be seen that far less replication is required at small dilution ratios than at higher dilution ratios for the same degree of accuracy. However, increasing the accuracy exacerbates the second problem of excessive use of materials.

Minaturization of dilution procedures, but not of the growth medium, was suggested by Fung and Kraft in 1968. Later Darbyshire *et al* (1974) developed a micromethod for determining the MPN of total bacterial and protozoan populations in soil using a small plate containing flat bottomed wells (capacity 0.35 cm^3) arranged in rows of eight wells by twelve wells. These microtitre plates, more frequently used by immunologists in the quantification of antisera, were tested for their suitability in the enumeration of nitrate-reducing and denitrifying bacteria.

TABLE 1

FACTORS FOR CALCULATING THE CONFIDENCE LIMITS FOR THE MOST PROBABLE NUMBER COUNT

Number of tubes per dilution	Factors for 95% confidence limits with indicated dilution ratios			
	2	4	5	10
1	4.00	7.14	8.32	14.45
2	2.67	4.00	4.47	6.61
3	2.23	3.10	3.39	4.68
4	2.00	2.68	2.88	3.80
5	1.86	2.41	2.58	3.30
6	1.76	2.23	2.38	2.98
7	1.69	2.10	2.23	2.74
8	1.64	2.00	2.12	2.57
9	1.58	1.92	2.02	2.43
10	1.55	1.86	1.95	2.32

2.1.4 Procedures

Pre-sterilized microtitre plates were taken and the base diluent, either nutrient broth or nutrient broth supplemented with 10 mM KNO_3 (see Appendix A), was distributed in 50 µL aliquots, using an Eppendorf Repeater pipette, into each of the 96 wells per plate.

Standard solutions of one Molar concentration sodium nitrate and sodium nitrite were prepared using analar quality chemicals (see Appendix A). An acidic, podzolic soil sample was taken, weighed and then mixed with sterilized phosphate buffer in a universal bottle to produce a one hundredfold (10^{-2}) dilution. Aliquots (50 µL) of standard solutions or soil suspension were pipetted into the first eight wells. Serial dilutions were then performed by using flame sterilized microdiluters, calibrated to deliver 50 µL. The microdiluter tips were placed in the 8 previously inoculated wells, rotated at least ten times in alternate directions, and then moved to the next 8 wells where they were again rotated rapidly. This process was continued until serial dilutions had been carried out across the entire plate resulting in 12 twofold dilutions with eight replicates at each dilution. In some cases the endpoint determination did not occur in the first plate and so a second plate was prepared in a similar manner except that well 1 contained the thirteenth dilution, through to well 12 containing the twenty-fourth dilution. The combinations of substrates and reagents tested are shown in Table 2.

TABLE 2

DETAILS OF SUBSTRATES AND REAGENTS USED IN A MODIFIED MICROTITRE/MPN PROCEDURE

Plate	Diluent 50 µℓ/well	Titrant	Bacterial inoculum present + absent -	Number of rows tested	Reagents used to test for Nitrite	Reagents used to test for Nitrate
A	nutrient broth	$NaNO_3$ standard	- -	4 4		25 or 50 µL Morgan's Zn/NEDD
B	nutrient broth	$NaNO_2$ standard	- -	4 4	NEDD 25 µℓ SA + 25 µℓ NEDD	
C	nutrient broth	$NaNO_2$ standard	-	4	50 µℓ SA + 50 µℓ NEDD	
D	nutrient broth	none	- - -	2 2 2	Zn/NEDD 50 µℓ SA & NEDD	Morgan's
E	NB + 10 $mMNO_3^-$	none	-	8		
F	NB + 10 mM NO_3^-	$NaNO_2$ standard	-	8	25 µℓ SA + 25 µℓ NEDD	Morgan's
G	NB + 10 mM NO_3^-	none	+	8	25 µℓ SA + 25 µℓ NEDD	Morgan's

NB = nutrient broth SA = sulphanilic acid Zn = zinc dust
NEDD = N-(1-napthyl) ethylenediamine dihydrochloride

Plate A was designed to compare the degree of sensitivity and ease of endpoint determination for the two methods used here to detect nitrate. Plates B and C acted in a similar capacity to A except that nitrite was under examination. The methods for the preparation of the chromogenic reagents are given in Appendix A. In order to determine whether nutrient broth affects any of the reagents, Plate D was set up. Plate E contained uninoculated growth medium in all 8 rows, where 4 rows were incubated with a covering and the other 4 rows remained uncovered. In this way the possible extent of aerial contamination, within the incubation chamber, could be ascertained. The effect of any interaction between the reagents used to detect both nitrate and nitrite was studied in Plate F. This was of particular importance as the test was designed to be a two reagent, simultaneous estimation. Plate G was used to test the procedures against a soil sample and to enumerate any nitrate-reducing or denitrifying organisms that might have been present. This experiment was repeated twice more to assess the reproduceability.

A dessicator was prepared as an anaerobic incubation vessel. Sterilized crucibles containing sterilized distilled water were placed on the bottom of the vessel to reduce evaporation of the small media volumes. The crucibles supported a perforated plate upon which the inoculated microtitre plates were placed. Two CO_2/H_2 producing BBL Gas Packs were placed in the centre and approximately 10 cm^3 of water added to each pack. A cold platinum catalyst was used to facilitate the reaction between hydrogen and any remaining oxygen to render the chamber anaerobic. The lid was sealed quickly and the dessicator partially evacuated to remove more oxygen. The anaerobic atmosphere became enriched with carbon dioxide and hydrogen as the Gas Packs worked. The lack of oxygen was demonstrated by the methylene blue anaerobic indicator changing from blue to colourless. The microtitre plates were incubated in the anaerobic chamber at approximately 20°C for seven days.

2.2 Sulphate-Reducing Bacteria

2.2.1 Microtitre/MPN Technique

For the detection and enumeration of sulphate-reducing bacteria a similar method to the one developed for bacteria involved in the reduction of nitrate was required. The difference is that the sulphate ion was to be monitored for its disappearance from a growth medium.

The medium used was a highly reducing medium necessary for the growth of these fastidious, anaerobic sulphate-reducing organisms (see Appendix A). Exclusion of air is not sufficient to ensure growth and hence a redox poising agent, thioglycollic acid, was used to give a value of approximately -220 mV. The total concentration of sulphate ion in the medium was 7.2 mM (693 mg/L SO_4^{2-}).

There are two methods for detecting and quantifying sulphate by visual observation, rather than the more generally accepted gravimetric methods. The methyl thymol blue method exhibits a pale blue colour in the absence of sulphate and a deeper colouration in the presence of the anion, so this method is only suitable for spectrophotometric determination. An alternative is a turbidometric method whereby the sulphate ion is precipitated in a hydrochloric acid and barium chloride medium so as to form barium sulphate crystals. Normally this increase in turbidity would be measured on a nephelometer. It was this method which was adapted to the microtitre/MPN technique. An acidic 'conditioning reagent' was prepared (see Appendix A) and 50 µL aliquots dispersed into each well. Using a micro spatula, a small amount of barium chloride crystals was added to each well and the contents gently mixed by moving the entire plate. The microtitre plates had been placed on a dark background to facilitate the detection of turbidity. All wells exhibiting turbidity were scored as positive for sulphate and hence negative for sulphate-reducing bacteria.

The concepts behind the modifications to the MPN technique (2:1:3) and the procedures described for bacteria involved in nitrate reduction (2:1:4) were the same for sulphate-reducing bacteria, with two exceptions. Due to the frequently lower viable counts for sulphate-reducers the soil sample was only diluted to a 10^{-1} level with phosphate buffer prior to inoculation. Only two plates were tested. Plate A contained phosphate buffer (50 µL/well) into which a standard sodium sulphate solution (1 M, see Appendix A) was serially diluted, using the microdiluters. Plate B contained the growth medium (50 µL/well) and was inoculated with the soil sample. Incubation was carried out under an anaerobic atmosphere for 7 days at 20°C.

2.2.2 Selective Medium/MPN Method

The more usual procedures for the MPN method were used in this instance, in combination with the use of a highly selective growth medium developed by Postgate (1979). This medium is a diagnostic one which contains a ferrous salt (0.05% w/v). Upon reduction of sulphate the hydrogen sulphide released reacts with the ferrous salt to produce a black precipitate. Blackening of the medium as a whole, or the zone around a colony, is evidence of bacterial sulphate reduction. Hence a sulphate-reducer code can be obtained which is converted to an MPN using a standard table.

A soil sample was diluted in phosphate buffer to give a tenfold dilution. This was repeated such that a series of tenfold dilutions from 10^{-1} to 10^{-5} was obtained. A 1 cm^3 aliquot of soil suspension was inoculated into a sterile test tube containing 6.0 cm^3 of liquid agar medium (held at 45°C). The contents were mixed and the agar allowed to set. The inoculated medium was overlayed with sterile liquid medium in order to exclude oxygen. The five, tenfold dilutions, with five replicates at each dilution, were incubated for up to 21

days at 30°C. This combination of dilution levels and replicates allowed reference to a standard MPN table prepared by Alexander (1965a).

3.0 RESULTS AND DISCUSSION

3.1 Nitrate-Reducing and Denitrifying Bacteria

After incubation the microtitre plates were placed on a white background to facilitate reading of results and the reagents added as shown in Table 2.

Addition of acidified diphenylamine (Morgan's reagent) resulted in a blue colouration in the presence of high standard nitrate concentrations. A 50 µL aliquot of the reagent resulted in a deeper blue colouration than the 25 µL aliquot. There was a progression in intensity of colouration becoming weaker as the degree of dilution increased. Visually detectable colour occurred in well 13 but not in well 14. The concentration of nitrate in well 13 was 0.000122 M which is equivalent to 7.564 mg/L of NO_3^-. When this was expressed in standard chemical terms, as weight of nitrogen in the nitrate ion, the value was 1.71 mg N/L.

The second method of detection used on Plate A involved the reduction of nitrate to the nitrite form using zinc dust and then determination of nitrite using N-(1-napthyl) ethylenediamine dihydrochloride (NEDD) reagent. Although this method has a high level of sensitivity when performed under chemically stringent conditions using accurate volumes of reagent, it did not work under the test conditions used in this work. It is known that the amount of zinc dust and the temperature are critical for this method and these may be the reasons for the lack of success.

In Plates B and C the presence of nitrite was observed by the production of a pink colouration in the case of the one reagent test (NEDD) and a deep magenta colour where two reagents were used. In the latter case the sulphanilic acid (reagent 'A', see Appendix A), reacts with nitrite to form a diazonium salt, which with the NEDD, forms a soluble red azo dye. The NEDD reagent alone could only be detected to well 10 whereas the sulphanilic acid/NEDD test produced faint colouration in well 20. In well 10 the Molar concentration is 0.000976 M, equivalent to 44.9 mg/L of NO_2^-, or 13.7 mg N/L. Whereas in well 20 the detectable weight of nitrite was 41.4 µg NO_2^-/L or 12.6 µg N/L. The use of sulphanilic acid gave a result that was an order of magnitude better than the single reagent determination. There was no appreciable difference between using 25 µL or 50 µL for the two reagent tests.

No colour changes were obtained in Plate D indicating that the bacterial growth medium, nutrient broth, did not produce any false positive results. On the other hand, the nutrient broth did not cause significant interference with the nitrite determinations as detection levels of 2 µg N/L are quoted in standard chemical tests (American Public Health Association, 1975), as opposed to 12.6 µg N/L obtained in this work. Extinction values for the colourimetric determination of nitrate using acidified diphenylamine have not been found in chemical tests, but Morgan (1930) reported the detection limit to be approximately 1 mg N/L which is similar to the result obtained in this study.

Plate E showed one contaminated well, that is visible signs of growth in the form of turbidity, out of the 4 rows incubated with a cover. The 4 rows incubated without any covering revealed three randomly distributed wells with signs of bacterial growth. As it is preferable to incubate the cultures open to the anaerobic atmosphere, it was decided to do so based on the low level of aerial contamination within the incubator.

For the experiment in Plate F the sulphanilic acid/NEDD reagents were added first for the detection of nitrite. The nitrite is then bound to a coloured complex and cannot interfere with the procedure for nitrate detection. The red colouration persisted for about 30 minutes. Upon addition of Morgan's reagent to the same wells, a faint to dark blue colour is formed at the interface of the reagent and the medium. Mixing of solutions by gently tapping the plate caused the faint blue colouration to disappear because the oxidation and benzene rearrangement necessary for the blue colour to exist only occurs at 70-80% H_2SO_4. Mixing obviously dilutes the acid. However, this was not so prevalent with higher nitrate concentrations where the blue colouration lasted for about 5 minutes. To overcome interpretation problems, the wells were scored for nitrate after the addition of reagent at one row at a time. Solutions in wells that were NEDD positive (nitrite present) always turned a deeper magenta colour when Morgan's reagent was added. The method selected for scoring the presence or absence of nitrate and nitrite was as follows. The sulphanilic acid/NEDD reagents were added to the entire plate and scored for the presence of nitrite. Then Morgan's reagent was added to a row of wells and if the blue colour persisted after this period of time it was designated as positive for nitrate. Each row of wells was recorded individually for the presence of nitrate in this manner.

The final plate type, G, was inoculated with a soil suspension and tested for the presence of nitrate-reducing or denitrifying bacteria. A diagrammatic representation of a typical inoculated microtitre plate is shown in Figure 1. Growth is recorded if the wells exhibit positive signs of growth in the form of turbidity. Wells that have

SERIAL DILUTION (TWOFOLD FROM INITIAL SUSPENSION IN WELL 1)

REPLICATES	WELL 1	2	3	4	5	6	7	8	9	10	11	12
A	0 *	0 *	0 *	1 *	0 *	1 *	1	2 *	2	1	2	2
B	0 *	0 *	0 *	0 *	1 *	1 *	1 *	2 *	2	2	2	2
C	0 *	0 *	0 *	1 *	1 *	0 *	1 *	1 *	0 *	2	2	2
D	0 *	0 *	0 *	1 *	2 *	1 *	1 *	1	1 *	2 *	2	2
E	0 *	0 *	1 *	0 *	1 *	1 *	1 *	1 *	1 *	2	2	2
F	0 *	0 *	0 *	0 *	0 *	1 *	1 *	1 *	2	2	1 *	2
G	0 *	0 *	0 *	1 *	1 *	1 *	1 *	1	1	2	2	2
H	0 *	0 *	0 *	0 *	1 *	0 *	2 *	2 *	2	0 *	2	2

FIGURE 1

DIAGRAMMATIC REPRESENTATION OF A TYPICAL INOCULATED MICROTITRE PLATE CONTAINING NUTRIENT BROTH PLUS 10 mM NITRATE

0 = NEITHER NO_2^- OR NO_3^- PRESENT 1 = NO_2^- PRESENT 2 = NO_3^- PRESENT * = VISIBLE GROWTH

160236 RD

no colour reaction after the addition of sulphanilic acid/NEDD and Morgan's reagent, were scored as positive for denitrifiers. Wells that turned pink with sulphanilic acid/NEDD were scored as positive for nitrate-reducing bacteria. Wells showing a blue colour indicated that the nitrate source remained untouched and that bacteria involved in the reduction of nitrate were not present at these dilutions. When considering the activity of bacteria able to reduce nitrate, it should be noted that the detection limit for nitrate was approximately 0.1 mM, 1% of the nitrate concentration (10 mM) in the growth medium. Therefore, about 99% of the nitrate must be depleted before any remaining nitrate becomes undetectable.

To translate colour reactions to Most Probable Numbers the following method was used. The dilution levels, (p) values, used to calculate the MPN were taken where: p_1 is the number of positive wells in the least concentrated dilution in which either all the wells are positive or the greatest number are positive, and p_2 and p_3 are the number of positive wells in the next two higher dilutions, respectively. Using this convention the code for denitrifiers in Figure 1 is well 2 $p_1 = 8$; well 3 $p_2 = 7$; well 4 $p_3 = 4$. A table of possible codes for the 12 dilution series with 8 replicates has been produced by Rowe *et al* (1977) and reference to this translates the denitrifier code of 874 to an MPN value of 1.855. The MPN value was then multiplied by the dilution factor for p_2 (8), corrected for initial dilution and soil moisture content to yield a value of 4.75×10^4 organisms/g soil (dry weight). The code for nitrate-reducing bacteria is well 7 $p_1 = 7$; well 8 $p_2 = 5$; well 9 $p_3 = 3$. The 753 code produced an MPN value of 0.992 which gave a viable count of 8.13×10^5 organisms/g soil (dry weight).

The frequency of visible growth in conjunction with the persistence of detectable nitrate was very low. Likewise, there were few wells that were scored positive for nitrate-reducers but showed no visible growth. It should also be noted that the pattern presented in Figure 1 is very typical as nitrate-reducing bacteria invariably had higher viable counts than denitrifiers. The values obtained in this investigation are consistent with other reported results. For example, Volz (1977) reported values of $1.4 - 3.0 \times 10^4$ denitrifiers/g soil as compared to 4.75×10^4 denitrifiers/g soil (dry weight) found in this study. Nitrate-reducers are normally present in higher numbers than denitrifiers as demonstrated by Volz (1977) who obtained numbers ranging from $8.5 \times 10^5 - 2.2 \times 10^7$ nitrate-reducers/g soil air dried, depending upon the soil type. The value reported herein of 8.13×10^5 organisms/g soil dry weight lies close to the documented range for nitrate-reducers.

3.2 Sulphate-Reducing Bacteria

The turbidometric method for detecting sulphate could be used despite the slight background cloudiness due to the growth medium. In Plate A the precipitate of barium sulphate crystals was observed up to well 13. This meant that the detection limit was at 0.000122 M Na_2SO_4 which converts to a value of 11.7 mg SO_4^{2-}/L. This is not as sensitive as the lower detectable limit of 1.0 mg SO_4^{2-}/L given by the American Public Health Association (1975) for this method.

When Plate B was examined for sulphate-reducing bacteria activity none was found. All the wells showed turbidity after reagent addition, not unsurprisingly as the sulphate concentration in the medium was 693 mg/L. A possible reason for this lack of growth was the small medium volume relative to surface area. In other words, despite the anaerobic atmosphere in the incubation chamber the medium did not remain sufficiently reduced for the growth of sulphate-reducers.

The selective medium/MPN method proved to be more successful. Blackening of the medium was observed after 10 days incubation. This blackening was so extensive that the tubes were scored as positive or negative for sulphate reduction, rather than counting individual colonies as a means of enumeration. The sulphate-reducer code was, well 3 $p_1 = 5$; well 4 $p_2 = 3$; well 5 $p_3 = 2$, which produced an MPN of 1.4 upon recourse to the tables. After accounting for the dilution level and soil wet weight the total number of viable bacteria was 2.24×10^4 cells/g soil dry weight. Other workers have reported values of 10^2 to 10^5 viable cells/g (Postgate, 1979) and 10^3 to 10^6 viable cells/g coastal sediments (Gunnarsson and Rönnow, 1982). There are few values for levels of sulphate-reducers in the literature but it would appear that the result obtained in this investigation falls within the accepted range.

4.0 CONCLUSIONS AND RECOMMENDATIONS

Successful isolation of both nitrate-reducing and denitrifying bacteria was obtained using nutrient broth supplemented with 10 mM nitrate. In the case of sulphate-reducing bacteria, the solidified (1.5% w/v agar) reducing medium (see Appendix A) containing 7.2 mM sulphate was optimal for their isolation. Therefore, both of these media were recommended for future use when studying bacteria involved in nitrate and sulphate reduction.

A modified microtitre/Most Probable Number technique was successfully developed for the enumeration of nitrate-reducing and denitrifying bacteria. The optimal parameters selected are described below.

Pre-diluted soil samples (50 µL) were inoculated into microtitre plate wells containing 50 µL of nutrient broth supplemented with 10 mM NO_3^- as the growth medium. Calibrated microdiluters were used to perform a two fold dilution series of twelve dilutions with eight replicates of each dilution. After one week of anaerobic incubation in a modified dessicator at 20°C growth had occurred.

Determination of the metabolic pathways utilized was performed by simultaneously detecting the presence or absence of nitrate and nitrite using a two reagent system. Nitrite was determined initially using sulphanilic acid (0.8% w/v) and NEDD reagent which produced a stable red azo dye if nitrite was present. The level of detection of nitrogen in the nitrite form was 12.6 µg N/L. An acidified diphenylamine reagent (50 µL) produced a blue colour in the presence of nitrate, with a detection limit of 1.71 mg N/L in the nitrate form.

Conversion of the positive and negative values for the anions into an MPN of bacteria was performed using a standard table. The numbers of denitrifying bacteria, 4.75×10^4 organisms/g soil dry weight, and nitrate-reducing bacteria, 8.13×10^5 organisms/g soil dry weight, obtained in this investigation were similar to reported values.

The modified microtitre/MPN method for the enumeration of sulphate-reducing bacteria was unsuccessful due to the lack of a sufficiently reducing environment to allow growth of these bacteria.

The selective medium/MPN method did allow growth of sulphate-reducers which produced an easily detectable sign, precipitation of FeS after 10 days. Conversion of the MPN gave a viable count of 2.24×10^4 cells/g soil dry weight. This result was in agreement with values given in the literature for these bacteria and therefore this method was recommended for the enumeration of sulphate-reducing bacteria.

Approved:

D. Harrison
Manager
Chemical Research Dept

Submitted:

V.J. Sharpe
Biologist
Biological Research Section

VJS:rdm

REFERENCES

1. Alexander, M. (1965a). Most Probable Number method for microbial populations. In Methods of Soil Analysis Part II (Black, C.A. *et al*, editors). American Society of Agronomy, Madison, Wis, USA pp 1467-1472.

2. Alexander, M. (1965b). Denitrifying bacteria. In Methods of Soil Analysis Part II (Black, C.A. *et al*, editors). American Society of Agronomy, Madison, Wis, USA, pp 1484-1486.

3. American Public Health Association (1975). Standard Methods for the Examination of Water and Waste Water. 13th Edition. American Public Health Association, Washington, USA, pp 455-458.

4. Bremner, J.M. (1965). Inorganic forms of nitrogen. In Methods of Soil Analysis Part II (Black, C.A. *et al*, editors). American Society of Agronomy, Madison, Wis, USA, pp 1179-1237.

5. Cochran, W.G. (1950). Estimation of bacterial densities by means of the most probable number. Biometrics, 6, pp 105-116.

6. Darbyshire, J.F., Wheatley, R.E., Greaves, M.P. and Inkson, R.H.E. (1974). A rapid micromethod for estimating bacterial and protozoan populations in soil. Rev Ecol Biol Sol, 11, pp 465-475.

7. Fung, D.Y.C. and Kraft, A.A. (1968). Microtitre method for the evaluation of viable cells in bacterial cultures. Applied Microbiol., 16, No 7, pp 1036-1039.

8. Gunnarsson, L.A.H. and Rönnow, P.H. (1982). Interrelationships between sulphate-reducing and methane producing bacteria in coastal sediments with intense sulphide production. Marine Biology, 69, pp 121-128.

9. Morgan, M.F. (1930). A sample spot-plate test for nitrate nitrogen in soil and other extracts. Science, 71, pp 343-344.

10. Postgate, J.R. (1979). The Sulphate-Reducing Bacteria. Cambridge University Press, Cambridge, UK.

11. Rowe, R., Todd, R. and Waide, J. (1977). Microtechnique for Most Probable Number Analysis. Appl Environ Microbiol., 33, No 3, pp 675-680.

12. Seeley, H.W., Jr. and VanDemark, P.J. (1981). Microbes in Action, 3rd Edition. W.H. Freeman and Co, San Francisco, USA, p 172.

13. Sirockin, G. and Cullimore, S. (1969). Practical Microbiology. McGraw-Hill, London, UK.

14. Valera, C.L. and Alexander, M. (1961). Nutrition and physiology of denitrifying bacteria. Plant and Soil, 15, pp 268-280.

15. Volz, M.G. (1977). Assessing two diagnostic methods for enumeration of NO_3^- reducing and denitrifying bacteria in soil-plant root associations. J. Soil Science Soc America, 41, pp 337-340.

APPENDIX A

MEDIA AND REAGENT COMPOSITION

Growth Medium for Bacteria involved in reduction of nitrate

Solution A - potassium nitrate solution (3.1% w/v)

potassium nitrate	3.10 g
distilled water	100.0 cm^3

Store +4°C

Solution B - nutrient broth

nutrient broth BBL No 2	2.5 g
distilled water	100.0 cm^3

Autoclave 115°C x 20 minutes

Solution C - nutrient broth with 10 mM KNO_3
To 100 cm^3 solution B add 2.0 cm^3 solution A.

Sulphanilic Acid (0.8% w/v) for the detection of nitrite

sulphanilic acid	0.80 g
5N acetic acid	100.0 cm^3

Store +4°C

NEDD reagent (0.6% w/v) for the detection of nitrite

N-(1-napthyl) ethylenediamine dihydrochloride	0.60 g
distilled water	99.0 cm^3
concentrated HCl	1.0 cm^3

If there is a precipitate filter. Store +4°C

Morgan's reagent (0.2% w/v) for the detection of nitrate

diphenylamine	0.05 g
concentrated H_2SO_4	25.0 cm^3

Cover bottle in silver foil, store +4°C.

Standard solutions

Nitrite	$NaNO_2$	6.9 g
	distilled water	100.0 cm^3
Nitrate	$NaNO_3$	8.0 g
	distilled water	100.0 cm^3
Sulphate	Na_2SO_4	14.2 g
	distilled water	100.0 cm^3

Growth Medium for Sulphate-Reducing Bacteria

Solution A - Salt Stock Solution (x 10 concentrate)

potassium dihydrogen phosphate (KH_2PO_4)	5.0	g
ammonium chloride (NH_4Cl)	10.0	g
sodium sulphate (Na_2SO_4)	10.0	g
calcium chloride ($CaCl_2 \cdot 2 H_2O$)	7.0	g
magnesium chloride ($MgCl_2 \cdot 6 H_2O$)	20.0	g
ascorbic acid	1.0	g
sterile distilled water	1000.0	cm^3

Store +4°C

Solution B - Stock Ferrous Sulphate Solution (x 10 concentrate)

ferrous sulphate ($FeSO_4 \cdot 7 H_2O$)	5.0	g
sterile distilled water	100.0	cm^3

Store +4°C

Complete Growth Medium (x 1 concentrate)

	For 1 litre		For 200 cm^3	
stock solution A	100.0	cm^3	20.0	cm^3
stock solution B	10.0	cm^3	2.0	cm^3
sodium lactate	3.62	cm^3	0.724	cm^3
yeast extract	1.0	g	0.20	g
Thioglycollic acid	77.0	µL	15.4	µL
Tap water	1000.0	cm^3	200.0	cm^3

Adjust pH to 7.6 with NaOH. Autoclave 115°C x 20 minutes
For solid medium Difco Bacto Agar to be added (1.5% w/v)
PRIOR to pH adjustment.

Conditioning Reagent for Sulphate Determination

concentrated HCl	6.0 cm^3
95% isopropyl alcohol	20.0 cm^3
sodium chloride	15.0 g
glycerol	10.0 cm^3

Store +4°C

POOR

QUALITY

ORIGINAL